2019-07

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10.1007/s00300-019-02522-3
Polar Biology
Springer Science and Business Media LLC

Erythrocytes nuclear abnormalities and leukocyte profile of the immune system of Adélie penguins (*Pygoscelis adeliae*) breeding at Edmonson Point, Ross Sea, Antarctica

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Abstract

Antarctic seabirds well adapted to extreme environments often deal during their life cycle with sub-optimal conditions and occasionally with severe environmental stress. Climate changes, pollution, habitat loss, increasing human presence can all significantly affect organism's health status from molecular to individual up to population level. In the present study, erythrocytes nuclear abnormalities (ENAs) and white blood cells (WBC) differential were investigated in 19 adults of Adélie penguin (*Pygoscelis adeliae*) breeding at Edmonson Point, Antarctic Specially Protected Area (ASPA n. 165) in the Ross Sea. Micronuclei (MN) accounted for 10.50% of observed abnormalities in penguin erythrocytes while kidney-shaped nucleus (KSN) was the most abundant (20.88%). Heterophils (HE) were the most common WBC (36.93%) in agreement with the generic avian leukocytes profile while eosinophils (EO) were the lowest (7.45%). A low number of lymphocytes were detected resulting in a higher heterophils to lymphocytes ratio. ENAs and H:L ratio are confirmed as reliable indexes of penguin's health status since they reflect their individual adaptation during breeding season. These baseline data will be useful for future studies as indicators of penguin's health status mainly as response to environmental changes.

Keywords: Adélie penguin, Antarctica, genotoxic damage, immune response, Ross Sea

Introduction

Organisms rarely experience optimal state in their natural habitat but for the most of their life they deal with variable conditions and occasionally with severe environmental stress. A variety of intrinsic factors can influence organism's physiological stress response such as reproductive status, age, sex, developmental or/and recent experiences. Whatever the source, physiological stress is a relevant parameter to consider when assessing animal welfare in both captive and wild populations (Davis et al. 2008).

Blood cells counts and classification, in particular erythrocytes' nuclear abnormalities (ENAs) and white blood cells (WBC), are considered efficient tools for assessing genomic instability and immune status in wildlife (Kursa and Bezrukov 2008). Although the mechanisms of the formation of ENAs are still little investigated in birds (Clark 2015), Van Ngan et al. (2007) promote the use of ENAs count for detecting genomic damage caused by prolonged exposure to several physico-chemical stressors during organism's lifespan. According to Kursa and Bezrukov (2008), occurrence of both micronucleus (MN) and in general nuclear abnormalities (NA) should be considered useful tools for assessing genome instability also in Antarctic birds since they represent a cellular reaction to natural and environmental stressors. MN occurrence may document what happen during erythrocytes' lifetime thus reflecting possible chronic effects. Furthermore, the white blood cell count (WBC) reflects animal's immune status and response to stressful conditions. The use of blood smears for detection of ENAs and WBC have several advantages such as low amount of blood needed with consequent low impact on animal health and a quick sampling procedure which can be readily used also in extreme environmental conditions as for instance with polar birds (Dantzer et al. 2014). Heterophils/ lymphocytes ratio (H:L) is considered a suitable indicator of organism's stress associated to reproductive cycle, seasonal changes, injury and also to pathogens and parasites (Dufva and Allander 1995; Krams et al 2012). Moreover, it reflects food and water deprivation, extremes temperature, constant light, long-distance migration and social disruption too. All these stressors result in an increased level of heterophils (innate immune system), decreased number of lymphocytes (acquired immune system) and a high H:L ratio (Vleck et al, 2000).

Commonly, birds exhibit low level of spontaneous blood cell anomalies such MN, therefore it might be rather easy to detect any alteration due genotoxicants exposure or other environmental stressors (Zúñiga-González et al. 2000, 2001).

Antarctic seabirds feed over wide geographical areas at different trophic level and therefore they are studied to monitor health conditions across large aquatic ecosystems and at different trophic levels. In turn they are able to reflect both natural and anthropogenic stressors (Mallory et al. 2010). Their health and physiological tolerance to stressors is closely influenced by their adaptation capability necessary to survive in their natural environment.

ENAs and immune status have been investigated in seabirds and in pygoscelid species breeding in the Sub-Antarctic and Antarctic Peninsula (Vleck et al. 2000; Kursa and Bezrukov 2008; D'Amico et al. 2014; De Mas et al. 2015; Barbosa 2013). ENA and immune status have been linked to contaminants exposure augmenting stress on penguin populations (D'Amico et al. 2014; Colominas-Ciuró et al. 2017) but also to

different stages of the breeding cycle, sex and individual condition and activities (Vleck et al. 2000; Moreno et al. 1998).

It is well known that climate changes are affecting the bioavailability of toxic contaminants in the wildlife altering the toxicokinetics due to an increase in temperature and salinity and leading to changes in organism' homeostasis and other physiological defence mechanisms (Noyes et al. 2009). Thus, during penguin's lifetime, contaminants exposure may vary according to the ecosystem changes. Different diets and foraging areas have been recognized also as major drivers for genome instability of penguin species from Antarctic Peninsula (De Mas et al. 2015).

In the background of above information, the present study investigates for the first time the occurrence of ENAs and WBCs in blood cells of an Adélie penguin (*Pygoscelis adeliae*, Hombron and Jacquinot 1841) population breeding at Edmonson Point, an Antarctic Specially Protected Area (ASPA n. 165) localized in the Ross Sea. The Adélie penguin is considered a keystone species of the Antarctic environment and currently most affected by environmental changes such as sea ice extent anomalies in different Antarctic regions (Ainley 2002; Olmastroni et al. 2004; Emmerson and Southwell 2008; Ropert-Coudert et al. 2013; Ducklow et al. 2013; Ballerini et al. 2009, 2015; Cimino et al. 2016).

In comparison with other Antarctic territories as for instance Antarctic Peninsula, the Ross Sea is still considered a pristine area (Halpern, 2008) and recently partially included in a Marine Protected Area (SC-CAMLR, 2016) to be preserved from increasing human activities. On the other hand, human pressure has increased significantly in the last twenty years mainly due to increase in fishing, tourism and number of scientific bases (De Mas et al. 2015; Tin et al. 2009). Scientific research communities strongly required protection for Antarctica from which the designation of the Ross Sea' MPA with the aim to preserve the marine ecosystem and biodiversity, as well as to limit and regulate current and future human impact. The Ross Sea is the home of 38% of the global population of Adélie penguin, therefore it is mandatory to address the current health status of population living in this territory in order to prove the efficacy of the MPA and to monitor any potential impact in the future. While ecology of Adélie penguin breeding at the Edmonson Point colony has been the focus of studies in the last 20 years (Olmastroni et al 2001, 2004; Pezzo et al 2007; Ballerini et al. 2009, 2015), genome and immune stability have not been investigated so far. This issue inspired our study on the occurrence of ENA and leukocyte profile of the immune system, with the aim to provide a baseline of health status of penguin living in the area.

Materials and Methods

Study area and samples collection

The penguin colony is located at Edmonson Point (74 ° 20' S, 165° 08' E), Ross Sea, an ice-free area of about 6 km^2 along the Eastern slopes of Mt. Melbourne and *c.* 50 Km NW from Mario Zucchelli Italian Research Station (Fig. 1). The area has been occupied by Adélie penguin (*Pygoscelis adeliae*) from almost 3000 years BP (Baroni and Orombelli 1994) and breeding population size consisted of 3066 pairs in 2014/15 summer season. Since 1994 Edmonson Point is a monitoring site to carry out scientific research on the

121 Adélie penguin's ecology and to collect data for the Ecosystem Monitoring Program (CEMP) lead by
122 CCAMLR Commission for the Conservation of Antarctic Marine Living Resources.
123 All data were collected during 2014-15 austral summer, following protocols approved by SCAR (SCAR's
124 Code of Conduct for the Use of Animals for Scientific Purposes in Antarctica, 2011) and under permission
125 from PNRA for working in an ASPA.
126 All blood and feather samples (19) were collected from adult penguins at the beginning of the breeding
127 season (incubation period) when mostly males occupy colony. Blood was collected from apparently healthy
128 penguins (i.e. not showing any sign of illness or injuries). No ectoparasites, feather or skin changes or
129 emaciation were observed.
130 In order to reduce stress induced by capturing and handling each bird was restrained for the minimum time
131 necessary (max 5 minutes) to carry out blood and feathers sampling and to record biometrics (Vleck et al.
132 2000). After sampling each bird was then released in front of its nest and observed until it returned to regular
133 breeding activity. Blood samples (one drop) were collected by venipuncture of the brachial vein using a
134 heparinized syringe with sterilized needle (22 gauge) according to Owen (2011). Up to five feathers per
135 individual were sampled from the chest area. Feathers were conserved in sealed plastic bags at -20°C.
136 Penguins were weighted with a Salter scale to the nearest 50 g, and bill depth and bill length measured using
137 a calliper. Blood smears were prepared in the field immediately after collection using a drop of blood on a
138 clean slide (15 min 10% HCl and rinsed with MilliQ water and oven-drying at 100°C). Slides were then
139 stored at +4°C in a slides' box.
140
141 **Genome and immune analysis**
142 Slides were processed at the University of Plymouth Ecotoxicology Lab for the analysis of genome
143 instability. The following procedure was used: slides were fixed using (100% v/v) cold methanol for 30 min,
144 stained with 10% Giemsa stain modified solution (Giemsa buffer tablets, pH 6.4 – BDH), and DPX
145 Mounting Media (Leica Biosystems). They were then observed under a light microscope equipped with
146 Digital Microscope Leica DMD108 Digital Microimaging Device with 40x objective. The images were
147 acquired, stored and processed by using LAS program (Leica Application Suite).
148 Areas with a clear distribution of erythrocytes were identified for each slide as a well-defined and separate
149 cytoplasm. Areas which presented overlapping cells were not taken into consideration.
150 Cell counting was carried out by taking the reference coordinates x, y and progressively moving from the left
151 to the right margin. Upon selecting the best images per slide, 1,000 erythrocytes were counted for each slide
152 according to Clark (2015) and the number of leukocytes and thrombocytes localized between them recorded.
153 A total amount of 19,000 cells was analysed.
154 In order to increase the identification of all known abnormalities in the nucleus of the erythrocytes of avian
155 species, in the present study blood cells of penguins were analysed according to the established method of
156 Kursa and Bezrukov (2008) already used for pygoscelid species by D'Amico et al (2014) and De Mas et al.
157 (2015). Erythrocytes nuclear abnormalities were determined as follows: (a) micronucleus, (b) lobed nucleus,

(c) tailed nucleus, (d) two-lobed nucleus, (e) budding nucleus, (f) nucleus with cavity, (g) kidney-shaped nucleus, (h) unknown nuclear malformation. Their sum as ENAs was also calculated.

White blood cells were classified along the five types of leukocyte according to Samour (2006): (a) heterophils, (b) lymphocytes, (c) monocytes, (d) basophils, (e) eosinophils were identified based on morphologic and staining characteristics according to the Table 22.10 reported in the chapter by Samour (2006). Erythrocytes and WBC were counted using ImageJ 1.6.0_24 (NIH, USA).

Sex determination

Sex of penguins (12 males and 7 females) was determined by molecular analysis on feathers except for one individual in which blood was used. DNA was extracted using the PureLink™ DNA Mini Kit (Invitrogen, by Thermo Fisher Scientific), following the manufacturer's instructions. The reliability of DNA extraction was monitored through a negative control (no tissue added), and the DNA content determined through an Eppendorf Ultraviolet Spectrophotometer (AG Eppendorf). The chromo-helicase-DNA-binding-1 gene (*CHD1*), found on sex chromosomes, was amplified which length varies among male (sex-chromosomes: ZZ) and female (ZW) penguins (Zhang et al. 2013). The following specific primers for penguins were used: PL (5'-CCC AAG GAT GAT AAA TTG TGC-3') and PR (5'-CAC TTC CAT TAA AGC TGA TCT GG-3'). PCR was run through a 2720 Thermal Cycler (Applied Biosystems), following this profile: 3 min 94°C, 30 cycles of 35" at 94°C, 45" at 55°C and 3' at 72°C, followed by 7 min at 72°C. PCR reactions were prepared with 0.5 µL of Taq Polymerase, 1 µL of each primer, 6 µL of PCR Master Mix (with PCR buffer, $MgCl_2$ and dNTPs: Genaid Biotech Ltd.) and about 20 ng of each DNA template. The electrophoresis was run for 45' on a 3% agarose gel (Zhang et al. 2013).

Statistical analyses

Descriptive statistical analyses including average, standard error (SE), range (minimum-maximum) of blood smear parameters were carried out with R Studio software (Version 0.99.902 – © 2009-2016 RStudio, Inc). Differences between sexes were determined through the nonparametric *Monte Carlo* exact permutation test for the equality of means, which computed all the possible permutations and uses the absolute difference in means as test statistic (Anderson 2001). The *Monte Carlo* exact permutation test assumes that the two samples are equal in distribution if the null hypothesis is true (Anderson 2001). Thus, we checked that each variable, both for male and female, followed the same distribution through a *Kolmogorov-Smirnov* test for equal distributions if the null hypothesis was true. Significance level was set at $\alpha = 0.05$. Analyses were performed through the software *Past* (Hammer et al. 2001).

Results

Erythrocytes nuclear abnormalities in the penguin's blood smears from Edmonson point colony are listed in Table 1. ENAs was found in 4.31 % over 19,000 mature erythrocytes analysed. Mean values of ENA varies from the lowest number of TLN (1.74 ± 0.40), which account for 4.03% of total ENAs to the highest of KSN (9.0 ± 1.14) accounting for 20.88% (Table 1).

194 Mean values of MN (4.53 ± 0.52) resulted similar to that found for lobed nucleus (LN) (4.79 ± 1.64) and
195 budding nucleus (BN) (4.79 ± 0.95) and account for 10.5% of total ENAs (LN and BN 11.11 %
196 respectively).
197 Therefore, the most recurring ENA are KSN, NWC and TN, followed by LN, MN and BN. Amongst those,
198 MN is the lowest abnormality occurring with ≤ 5 over 1,000 mature erythrocytes while NWC, TN, LN e
199 KSN exhibited higher variability. In addition, a small percentage showed unknown nuclear malformation
200 (UNM) (Table 1). The figure 2 shows all ENAs detected in Adélie penguin's blood smears classified
201 according to Kursa and Bezrukov (2008) and De Mas (2015).
202 Table 2 summarizes WBC identified in Adélie penguin's blood samples. White blood cells were 658 over
203 19,000 cells scored in penguin's blood smears. Heterophils (HE) were the most common WBC, followed by
204 lymphocytes (LY), basophils (BA), monocytes (MO) and eosinophils EO (Table 2 and shown in Fig. 2).
205 Although not significant, toxic HE (THE) resulted higher than normal HE (NHE) in the total HE found (243
206 over 19,000 erythrocytes scored). LY resulted lower than total HE (23.70% compared to 36.90%) while BA
207 were 19.45% of the total WBC (Table 2 and Fig. 3).
208 Total MO resulted 12.46% of the total leukocytes over 19,000 cells scored. Mean TMO numbers resulted
209 higher than NMO even though not significantly different. The lowest WBC (Fig. 3) detected were EO
210 (7.45%). Heterophil: Lymphocyte ratio (H:L) was calculated and the mean value was 3.08 ± 0.87.
211 The number of HE (*Monte Carlo* exact permutation test: $p = 0.016$) and the number of NHE (*Monte Carlo*
212 exact permutation test: $p = 0.012$) were approximately four times greater in males (n = 12) than in females (n
213 = 7) (Fig. 4), the rest of parameters analysed showed not gender differences.
214

215 Discussion

216 The present study investigates for the first time the occurrence of ENAs and WBCs in blood cells of Adélie
217 penguin (*Pygoscelis adeliae*, Hombron and Jacquinot 1841) breeding at Edmonson Point, an Antarctic
218 Specially Protected Area (ASPA n. 165) localized in the Ross Sea.
219 The most frequent ENAs described for bird populations including penguins have been observed in blood
220 smears of Adélies from Edmonson Point (Kursa and Bezrukov 2008 and De Mas 2015); in particular
221 peculiar nuclear anomalies as TN, KSN and TL were observed (Lucas and Jamroz, 1961) as well as BN and
222 LN which are considered in interphase as precursors of MN formation and associated to cell death, genomic
223 instability, or cancer development (Webster et al. 2009).
224 MN frequency is also in the range of natural values reported for birds (from 0.40 to 4.30 over 1000
225 erythrocytes scored) (Zúñiga-González et al. 2001), thus suggesting a low genome instability of individual
226 nesting at the Edmonson Point colony.
227 The analysis of immune parameters also reveals that total number of WBC are within the normal range
228 reported for birds (Kursa and Bezrukov 2008). By comparing ENAs and H:L ratio observed in Adélies from
229 Edmonson Point with those documented in penguins breeding in Antarctic Peninsula (i.e. more
230 anthropogenically impacted: Tin et al. 2009; SCAR 2010), some considerations can be made.
231 ENA values result similar to those reported by De Mas et al. (2015) in Adélie penguin from Torgensen and
232 Avian Islands (43.11 ± 27.59; 46.90 ± 46.50; 41.20 ± 40.10 respectively) whereas those of penguins from
233 Yalour and King George Island result far higher (109.90 ± 80 and 72 ± 35.3). Lower values are on the
234 contrary reported by D'Amico et al. (2014) in penguins from Potter Peninsula at Stranger Point (26.20 ±
235 3.20) scoring 40,000 mature erythrocytes out of 20 individuals.
236 MN values (4.53 ± 0.52) are similar to the range reported in penguins breeding in colonies located in the
237 Antarctic Peninsula (Yalour Island, 5.2 ± 4.1; Avian Island, 3.25 ± 3.7) but higher than those recorded in
238 penguins from Torgensen Island (1.3 ± 1.5) and King George (1.9 ± 1.4) (De Mas et al. 2015).
239 Interspecific comparison among *Pygoscelis* genus, shows lower MN values in individuals of *Pygoscelis*
240 *papua* (Gentoo penguin) and *Pygoscelis antarcticus* (Chinstrap penguin) (De Mas et al. 2015 and reference
241 within) compared to Adélies from Edmonson Point (this study).
242 Several ecological and environmental factors, such as species-specific sensitivity, diet, wintering areas and
243 exposure to toxic pollutants, could affect penguin's genome and immune stability (Bargagli 2005; Barbosa et
244 al. 2013; De Mas et al. 2015). According to De Mas et al. (2015), a different sensitivity to environmental
245 disturbance of Gentoo and Chinstrap penguins compared to the strictly sea ice dependent Adélie penguin,
246 might have resulted in the development of a physiological defence mechanism able to cope better with
247 genotoxic agents. In addition, it has been hypothesized that some of the observed differences among species
248 could be related to the diet spectrum, which is wider in Gentoo penguins compared to Adélies (D'Amico et
249 al. 2016). D'Amico et al. (2016) address also anthropic sources as responsible of observed ENAs recorded in
250 Adélie penguins from Stranger Point where high levels of heavy metals (Ni, Cu, Zn ad Se) have been
251 detected in their feathers. Ancora et al. (2002) reported heavy metals (Cd, Pb and Hg) in stomach contents,

252 excreta, and feathers of Adélie penguins breeding at Edmonson Point. At that time a natural occurrence has
253 been hypothesized for Cd and, to a lesser extent, for Hg, but not a direct anthropogenic impact of local
254 sources. In fact the nearest scientific stations are far (*c*. 50 Km) from the Edmonson Point colony.

255 Concerning other source of anthropic pollution, contaminants stored in pack ice during years (via global
256 distillation process), could be released as a result of the seasonal melting also amplified by increasing
257 temperatures of surface waters as a consequence of climate changes (SCAR 2010). For instance, Persistent
258 Organic Pollutants (POPs) have been documented to cause alteration on immune system (Jara et al. 2018)
259 and to correlate with alterations in ENAs and WBC in penguin's species (Jara-Carrasco et al. 2015). In
260 Adélie penguin population breeding at Edmonson Point, legacy POPs have been reported in stomach
261 contents, blood samples and unhatched eggs by Corsolini et al. (2003, 2011, 2017), but overall toxicity was
262 estimated to be low compared to other Antarctic areas. Emerging contaminants like PBDEs (Corsolini et al.
263 2017) and PFAS (Ademollo, unpublished data) were also detected in Adélie penguin blood samples and eggs
264 from Edmonson Point. Therefore exposure to contaminant in penguins breeding at Edmonson Point cannot
265 be considered negligible; Antarctic penguin's colonies are also considered a secondary source of POPs
266 (Roosens et al. 2007). Nonetheless the impact of human activities that determines local inputs need further
267 investigations (Wang et al. 2017). A small seasonal field camp (average of 2 personnel unit) located 600 m
268 far from the breeding groups represents so far the only local source of contamination at Edmonson Point
269 (Olmastroni 2002).

270 As far as immune status parameters, mean H:L value results higher compared to those reported by D'Amico
271 et al. (2014; 2016) in Adélie penguin from Stranger Point (1.10 ± 0.20 and 1.07 ± 0.11 respectively). In
272 particular, the percentages of LY, HE and EO result lower than those reported by D'Amico et al. (2014)
273 while MO and BA are 37% higher. MO and EO can be used to make a distinction among factors that alter
274 the leukocyte profile: stress, disease and infection. In fact, MO number increases in case of infections and
275 diseases since their main role is to phagocyte foreign particles. On the opposite, a reduction in EO number is
276 commonly a measure of stress reaction and rarely a response to disease. Early studies in human and
277 mammals confirmed that glucocorticoids induced by stress often carry out a reduction on EO numbers
278 (Davis et al. 2008 and references within). THE were also detected and described by Jara-Carrasco et al.
279 (2015) as a cytological alteration consequent to exposure to various stress agents. THE in Adélie penguins
280 accounted to 53.50% of total HE and may suggest a bird's response to stress. The presence of THE
281 associated with the abnormal high number of BA identified in Adélie penguin's blood smears may indicate
282 some disease occurring in the population under study. Mild lymphocytosis and moderate basophilia have
283 been associated with feather loss in penguins population from the Ross Sea (Grimaldi et al., 2014) which has
284 been lately observed also in individuals from Edmonson Point in a similar percentage of occurrence
285 (Olmastroni personal. observation, 2018-19 Antarctic expedition).

286 Concerning WBC, higher values are reported by D'Amico et al. (2016) in Adélie penguin from different
287 Islands around Antarctic Peninsula. However, among them, similar values as those measured in our study

were reported in Adélie penguins from Stranger Point in which in a comparable number of individuals was analysed (n = 20). HE shows the highest percentage and this type of WBC are phagocytic cells that increase when the organism needs to cope with infections causing an increase in the level of H:L ratio. For instance, HE are the first line of defence that an organism uses as immune response against gastrointestinal parasites incorporated through the diet (D'Amico et al. 2016). An organism affected by heterophilia and lymphopenia presents the same leukocyte profiles as one who is experiencing infection and/or diseases. In addition, despite anthropogenic pressure may have a strong influence on H:L ratio, this factor might have had less influence in penguins monitored in the present study since penguins from Edmonson Point colony seem less affected by organic pollutants compared to other colonies (Schiavone et al. 2009).

Although difficult at this stage to connect to any contamination or stress sources, this information will be helpful for future investigation for comparison with different seasons, colonies and breeding stages. In addition, some aspects of the breeding ecology need to be considered for assessing the health status of the penguin population. During the breeding stage, females usually arrive later at the breeding colonies (Ainley 2002), and fasting period and intraspecific competition are reduced if compared to mates. At the time of sampling adults were incubating eggs or attempting to breed, according to Edmonson Point breeding chronology (Olmastroni et al. 2000; Pezzo et al. 2007). Consequently males were fasting from their arrival at the breeding colony (late October) and underwent competition with conspecifics for territory occupation, nest building and mating. Thus, reproductive cycle, seasonal changes, fasting, long-distance migration, competition for resources and injuries can all affect health status e.g. H:L ratio (Moreno et al. 1998; Vleck et al. 2000; Minias 2019). Seasonal changes may influence organism's stress levels forcing individuals to use more energy for thermoregulation. Vleck et al. (2000) reported that injured birds during fights for defending their territory and/or nest, exhibit higher H:L ratio level than healthy birds. In addition, pathogens, ecto and endoparasites are known to affect immune status. Individuals sampled in the present study were healthy penguins, as their weights ranged 3100-5650 g, no sign of illness or injuries, and no ectoparasites, feather or skin changes or emaciation were observed. There are no studies available on pathogens or parasites on Edmonson Point population. We cannot exclude potential influence of disease or parasites hampering health status in the studied population, but no evidence of blood pathogens was detected in the current study. In addition, studies on pygoscelids suggested absence of blood parasites and a low richness of ecto and endoparasites for wild sub-Antarctic and Antarctic species (Jones and Shellam1999; Diaz et al. 2016; Vanstreels et al. 2014, 2016).

Environmental natural stressors and increasing anthropogenic impact on wildlife are expected to grow in Antarctica in the near future, potentially by altering individual's level of stress and immune status. The present results depict a preliminary overall assessment of the health status of Adélie penguin's colony at Edmonson Point since it reflects the different components of an organism's response to its environment. ENAs and H:L ratio parameters represent a first baseline for future monitoring and assessment of genome and immune stability of Adélie penguin population in the mid Victoria Land area. Because high H:L ratio may represent a corticosterone-mediate response of organism to various exogenous stressors and an adaptive

evolutionary trait (Minias 2019) future investigation and sampling will be carried out in the framework of the ongoing research program PNRA2016 AZ1.11 (PenguinERA). Blood parameters such as estimations of ENAs, WBC and H:L could be useful physiological and ecological indicators in monitoring and conservation studies to assess population and ecosystem health in a changing environments.

Acknowledgements

The authors thank the PNRA for logistical support during the XXX Italian Antarctic expedition.

We also thank Dr Lorna Dallas and Dr Will Vevers from Plymouth Laboratory for helping with blood cells analyses. We thank reviewers, Veronica D'Amico and Luciano Calderon, for their criticisms and precious comments which significantly improve the quality of the manuscript.

Fundings

This research was funded and carried out in the framework of Project PNRA2013 AZ1.22. *Conservation of a mesopredator sensitive to environmental changes*.

Author's contribution

SO and IC conceived of this study and wrote up the manuscript, IC and ANJ planned genome and immune lab analyses, SO and NA collected data, GP performed genome and immune lab analyses, EM and MLV performed molecular lab analyses, NF performed statistical analyses. All authors helped to draft the manuscript, read and approved the final manuscript.

Ethical approval

All applicable international, national and /or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of SCAR's Code of Conduct for the Use of Animals for Scientific Purposes in Antarctica.

Conflict of interest

We declare that we have no conflict of interest with the data presented in this scientific contribution.

Figure and table captions

Fig. 1 Adélie penguin colony at Edmonson Point (74°20' S, 165°08' E), Victoria Land, Ross Sea.

Fig. 2 Erythrocytes nuclear abnormalities (ENAs) in Adélie penguin blood samples according to Kursa and Bezrukov (2008): (a) micronucleus (MN), (b) lobed nucleus (LN), (c) tailed nucleus (TN), (d) two-lobed nucleus (TLN), (e) budding nucleus (BN), (f) nucleus with cavity (NWC), (g) kidney-shaped nucleus (KSN), (h) unknown nuclear malformation (UNM).

Fig. 3 Differential white blood cells (WBC): (a) Heterophil (HE), (b) Toxic Heterophil (THE), (c) Lymphocyte (LY), (d) Monocyte (MO), (e) Toxic Monocyte (TMO), (f) Basophil (BA), (g) Eosinophil (EO).

363 **Fig. 4** Mean ± standard error of n. of heterophils and n. of normal heterophils counted in males and females
364 Adélie penguin (females: n = 7; males: n = 12)

365 **Table 1** Number of micronucleus (MN) and other erythrocytes nuclear anomalies (ENA) analysed per
366 19,000 mature erythrocytes of Adélie penguin's blood smears according to Kursa and Bezrukov (2008), and
367 to De Mas (2015)
368 **Table 2** White blood cells (WBC) per 19,000 mature erythrocytes in Adélie penguin's blood samples and H:
369 L ratio
370

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